Novel Computer Threshold Analysis for Quantification of Inflammation and Fibrosis in Renal and Cardiac Tissue in Immunohistochemistry Images

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Introduction:

Renal Disease:

More than 2 out of every 1000 people are affected by kidney disease, a serious condition in which the kidneys fail to clear wastes from the body (2). As with other organs in the body, the kidney's function can decrease with age (5). As the kidneys age the number of nephrons and overall amount of kidney tissue decrease and the blood vessels supplying the kidney harden. As a result, the glomerular filtration rate decreases meaning the kidneys filter blood more slowly. Increased inflammation and fibrosis occurs in aging kidneys and can be detected in research experiments through the use of immunohistochemistry marking macrophages and collagen, respectively (18). More specifically kidney disease can be either acute or chronic with kidney failure defined within the final stage of chronic kidney disease. Kidney failure is when the kidney is working less than (1/10) one-tenth of its normal function.

The two key markers for chronic kidney disease are the estimated glomerular filtration rate (eGFR) and urine albumin. The calculation of eGFR is based on the amount of creatinine, a waste product, found in a blood sample and as the creatinine level increases, eGFR decreases (6). Serum albumin is a protein produced in the liver. The blood concentration of albumin decreases with inflammation due to a decrease in production while the urine concentration of albumin increases due to damage in the filtration system of the kidney. Albumin should never be present in urine and even a small increase in the ratio of albumin to creatinine is a sign of kidney damage. Kidney disease is diagnosed when the eGFR is less than 60 mL per minute or when urine contains more than 30 mg of albumin per gram of creatinine.

Diabetes is the leading cause of chronic kidney disease in the United States. About 30 percent of patients with Type 1 diabetes and up to 40 percent of patients with Type 2 diabetes eventually will suffer from kidney failure. Menopause can have a profound effect on non-diabetic related kidney disease in women

because premenopausal women with non-diabetic kidney disease show slower rates of progression compared to age-matched men, a difference that disappears after menopause (17). The hormone levels in women are thought to have an effect on the progression of non-diabetic kidney disease in post-menopausal women (14). However, in diabetic renal disease, the effect of gender has not yet been determined (21).

Rapamycin Treated Old Animals with Chronic Kidney Disease:

Rapamycin is an anti-proliferative, immunosuppressant, and anti-cancer drug agent (7). This experiment was completed to study whether rapamycin could help rescue age-related kidney disease by decreasing activity of the mTOR (mammalian Target of Rapamycin) pathway that is responsible for translation, metabolism, autophagy, ribosome biogenesis, and senescence (13).

For this study 18 month-old old mice were compared with 12 week-old adult mice. There were 16 mice in each of the 4 treatment groups per age: control, metformin (200 mg/kg), low dose (75 μg/kg) rapamycin, and high dose (2.5 mg/kg) rapamycin. All of the metformin mice died during the study due to the high toxicity of the drug. Baseline weights and urine osmolalities were measured prior to injection of the drugs and all the kidneys were harvested after 3 months of daily injection.

Diabetic Kidney Disease in VCD Model of Menopausal Mice:

In this study, injection of 4-vinylcyclohexene diepoxide (VCD) toxin in mice induced ovarian failure by causing follicle atresia. The mice entered menopause, ovarian failure, earlier than they would have normally (11). VCD was administered via intraperitoneal injection at a dose of 160 ml/kg body weight using a dosing standard of 2.5 ml/kg body weight for 15 consecutive days to induce ovarian failure. Progression into ovarian failure was monitored by daily vaginal cytology. Ten consecutive days of diestrus was indicative of ovarian failure (16).

To address the impact of post-ovarian failure on the development of diabetes and subsequent kidney damage, streptozotocin (STZ) was injected to induce diabetes in the VCD model of menopause (11). STZ-induced diabetes was begun during the period analogous to post-menopause in humans (post-ovarian failure). Once enough mice reached diestrus (28 mice per time-point) the diabetic and diabetic menopausal groups were dosed with STZ and the groups were sacrificed at 3 different time-points: 6, 8, and 12 weeks post-STZ injection. Diabetes was induced by intraperitoneal injection of STZ at a dose of 0.2 ml/g body weight to 4-hour fasted mice for 3 consecutive days.

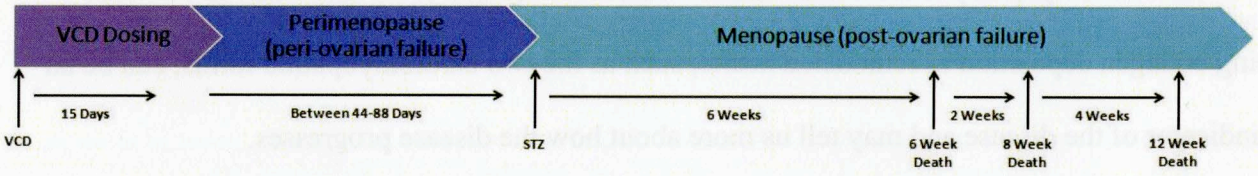


Figure 1: Timeline of experimental study groups. All mice were dosed for 15 consecutive days with 4-vinylcyclohexene diepoxide (VCD) to induce gradual ovarian failure. 10 consecutive days of diestrus was indicative of ovarian failure and the first, second, and third group of mice were dosed with streptozotocin (STZ) for 3 consecutive days then sacrificed at 6, 8, and 12 weeks past the last STZ dose, respectively.

It was hypothesized that changes in hormone levels post-menopause will increase the development of diabetes and the negative effects of diabetic kidney damage (11). Prior to quantitative analysis, Figure 2 shows a qualitative observation that menopausal diabetic mice express higher levels of invading macrophages in renal tissue indicative of higher levels of renal disease.

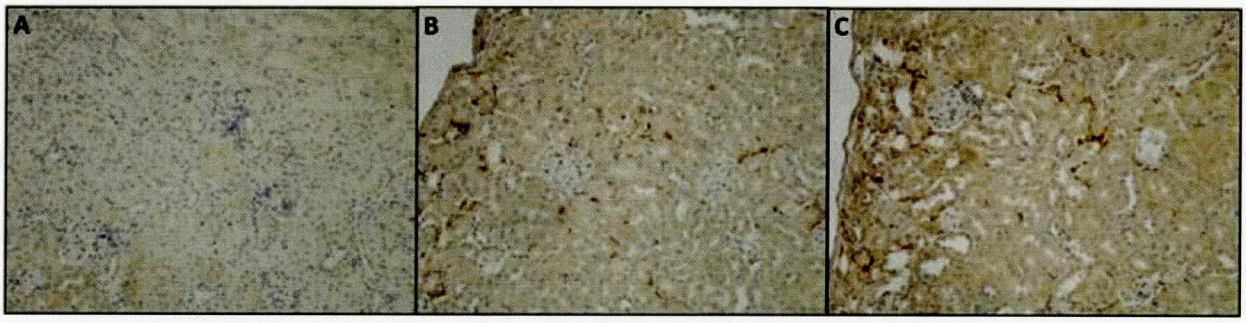


Figure 2: F4/80 immunohistochemistry images pre-quantitative analysis process of VCD/STZ Mouse study. A: Control group renal tissue; no positive DAB staining for F4/80. B: Diabetic group renal tissue; very little positive DAB

staining for F4/80 antigen. C: Menopausal diabetic group renal tissue; apparent increase in positive DAB staining for F4/80 antigen is seen here.

Hypertrophic and Dilated Cardiomyopathy:

Cardiomyopathy means diseased heart muscle. Two forms of cardiomyopathy are the hypertrophic cardiomyopathic hearts when the muscle around the heart thickens and the other form is dilated cardiomyopathic hearts when the muscle surround the heart thins and weakens. Often hypertrophic cardiomyopathy will progress into dilated cardiomyopathy (15). People diagnosed with hypertrophic cardiomyopathy are at a higher risk for sudden cardiac death than the normal population (24). Currently, the cause for cardiomyopathy is somewhat unknown and it is thought to be hereditary in humans. Studying collagen deposition in remodeled hearts, such as the two cardiomyopathic forms, can be an early indicator of the disease and may tell us more about how the disease progresses.

Two-Mutant Crossed Cardiac Mouse Model:

Mutations in sarcomere protein genes can cause hypertrophic cardiomyopathy (HCM); a disorder characterized by myocyte enlargement, fibrosis, and impaired ventricular relaxation. A transgenic mouse line was created with a single allele mutation at R403Q, where the arginine at position 403 was replaced with glutamine, in the actin-binding domain of the myosin heavy chain (MyHC) in cardiac muscle tissue (23). The function and amount of the α-MyHC in mice are comparable to the β-MyHC found in humans. The transgenic mice present with hypertrophic cardiomyopathy and increased energetic strain at 12 weeks-old (22).

Another transgenic mouse deleted the subunit 7a of cytochrom c oxidase (COX), isoform COX7a1, the major cardiac form. The homozygous and heterozygous knockout mice for the COX7a1 deletion display reduced COX activity and develop dilated cardiomyopathy by 6 weeks-old. However, the disease

stabilizes and improves by the time the mice are 6 months-old (8). These knockout mice do not develop hyptertrophic cardiomyopathy but instead progress directly to dilated cardiomyopathy.

Immunohistochemistry:

Immunohistochemistry (IHC) refers to the process of detecting antigens such as proteins in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues (4). IHC helps researchers and pathologists make somewhat subjective observations of *in situ* protein expression. IHC takes its name from the roots "immuno," in reference to antibodies used in the procedure, and "histo," meaning tissue. IHC is also widely used in basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue. Visualizing an antibody-antigen interaction can be accomplished in a number of ways. In the most common instance, an antibody is conjugated to an enzyme, such as peroxidase, that can catalyze a color-producing reaction.

For studies related to this work, macrophages were the tissue antigen and the antibody F4/80 was employed to bind to the F4/80 antigen on the macrophages. A species-specific secondary antibody was then applied that bound to the primary antibody. Diaminobenzidine (DAB) is the reporter molecule that bound to the secondary antibody and was used to visualize the macrophages in the tissue. DAB was used instead of fluorescent reporter molecules because the fluorescently tagged antigens when imaged display as black or white with varying intensity. This drastically reduces the user's ability to distinguish between positive staining versus a high background color. Using a brown color as a marker the user has more control to objectively quantify the immunohistochemistry stain results.

Picrosirius Red Stain:

Sirius Red, a strong anionic dye, stains collagen by reacting, via its sulphonic acid groups, with basic groups present in the collagen molecule (10). The elongated dye molecules are attached to the collagen fiber in such a way that their long axes are parallel. This parallel relationship between dye and collagen results in an enhanced birefringency (12). Birefringence is when a light wave is split into two unequally reflected beams, each refracted at a different angle, and each beam polarized at a right angle to the other. If a substance is birefringent then when polarized light travels through it, the vertically polarized light will travel more slowly but at the same frequency compared to the horizontally polarized light which will not be delayed (1). In essence, under polarized light, the birefringent substances will reflect one beam of light and the collagen will appear red (9). By using Picrosirius red to stain collagen in tissue, we can visualize the amount of collagen deposition in early disease states in both renal and cardiac tissues.

Current Immunohistochemistry Analysis:

Although there is a long history of efforts to quantify immunohistochemistry, there has been a lack of broad acceptance because the resultant objective accuracy has not significantly improved outcome measures compared with the traditional, qualitative, conventional analysis by eye (4). The current processes and software for assessment of *in situ* protein expression are manufactured for large-scale throughput like hospitals, are costly, and not reliably accurate (4). There are other processes designed by academic institutions that focus on specific biomarkers or only the protein of interest for their application. Currently, the method for data analysis of immunohistochemical stains involves subjective perception of the amount of brown DAB positive stain is present in a slide of one experimental group versus another group. This qualitative process does not allow for quantitative data collection or statistical analyses. There is a need for an efficient, accurate, inexpensive, and non-subjective analytic

method to quantify the level of protein expression in immunohistochemistry images that can be applied to a variety of antibodies and protocols.

ImageJ:

ImageJ is a public domain, Java-based image processing program developed at the National Institutes of Health and is easily downloadable to any computer. The program is straightforward and can handle many different image files – for the purposes of this work .jpeg and .tiff were used (3). ImageJ is a great tool for image processing and accessible for users unfamiliar with programming and analysis.

Methods:

F4/80 Immunohistochemistry:

Kidneys were fixed in 4% paraformaldehyde overnight, embedded in paraffin and sectioned at 4 μm thickness by the University of Arizona Pathology Laboratory. Sections were deparaffinized in xylene overnight and rehydrated in gradations of ethanol. Endogenous horseradish peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 15 minutes. Antigen retrieval was performed in the 1 mM citrate buffer at 95˚C for 20 minutes. Nonspecific binding was blocked with 5% goat serum in 2% bovine serum albumin (BSA). Tissue sections were incubated overnight at 4˚C with a macrophage tagging antigen (F4/80; 1:20 dilution), followed by a secondary antibody for specificity at 37˚C for 30 minutes. Labeling was visualized with chromogen diaminobenzidine (DAB) for 5 minutes and counterstained with hematoxylin for 1 minute. Coverslips were mounted with Permount mounting solution and analyzed with the ImageJ threshold process.

Microscope Settings:

Quantification of DAB requires that the specific, positive staining stands out from non-specific background staining. For ImageJ to accurately analyze immunohistochemical staining, images are taken under white light set at maximum intensity to dilute the non-specific background staining. Images were taken at 40x magnification.

NIS Elements Camera Software Settings:

Three adjustable parameters on the NIS Elements camera program are exposure time, gain, and contrast. The "Auto-white" function was not used in these studies as it brings the non-specific background staining into focus. Exposure time is the effective length of time that the camera's shutter is open. Gain electronically brightens an image. Contrast is the difference between the light and dark areas in an image; increasing contrast can help the specific staining stand out from the non-specific background staining (20). The three types of contrast in this process that are of interest for this application are high, enhanced, and anti-reflex contrast.

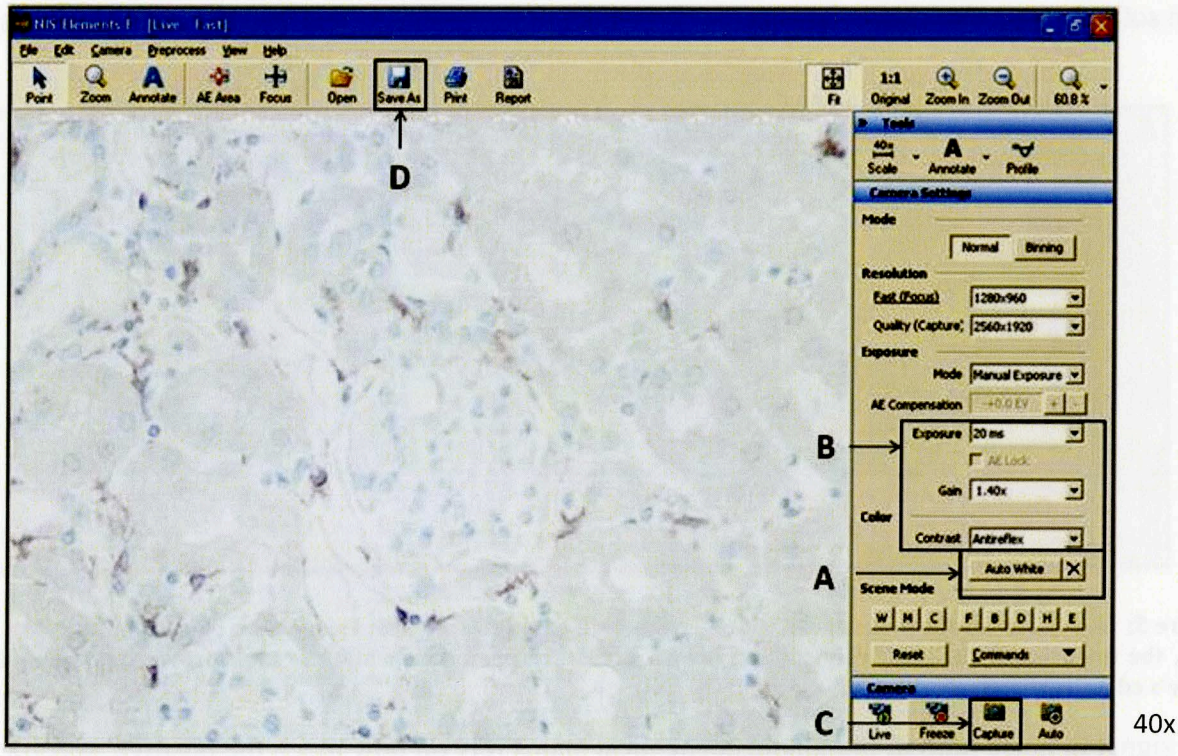


Figure 3: Screenshot of NIS Elements camera program. A:"Auto-White" not used in process .B: Camera settings that user adjusts: exposure, gain, and contrast. C: "Capture" button to take picture. D: "Save As" button to save image as a .jpeg file.

Each application of the ImageJ Threshold analysis to a full IHC run required the user to test whether enhanced, high, or anti-reflex contrast worked best with the power of the stain and the background color. For the purposes of this work, several iterations of this process proved that the anti-reflex contrast is best. The following images illustrate the differences between the 3 contrast types with an exposure time of 20 µs and gain of 1.40:

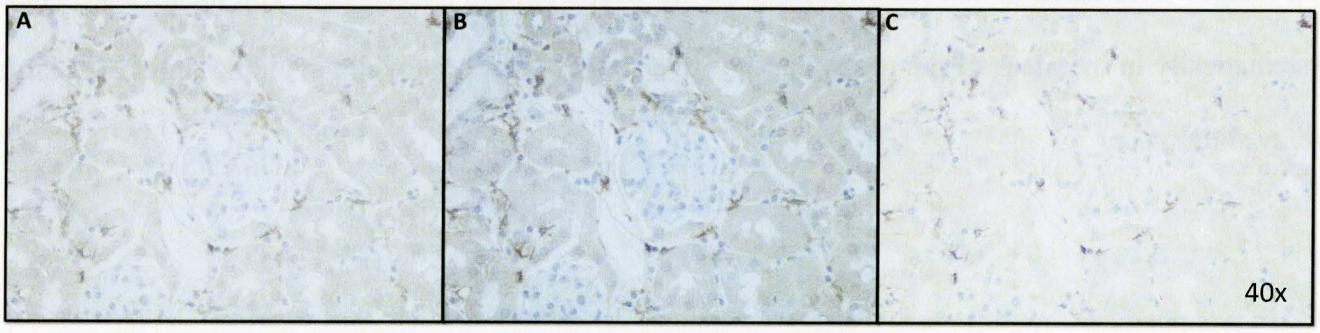


Figure 4: Panel displaying differences in contrast filters with gain equal to 1.40x. A: High Contrast. B: Enhanced. C: Anti-reflex.

Gain set at 1.40x helps dilute the background color while preserving the integrity of the brown stain:

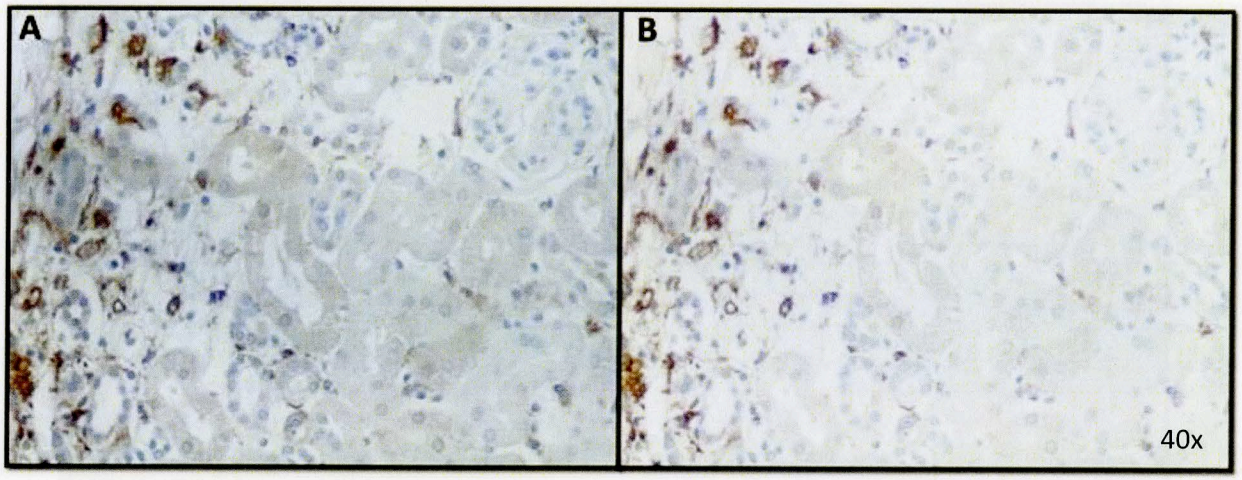


Figure 5: How Gain affects an image. A: Image with gain equal to 1.20x. B: Image with gain equal to 1.40x. Note that in B, the intensity of the positively stained brown areas are preserved while the high brown background near the tissue's edges is effectively diluted.

The signal to noise ratio in immunohistochemical stains rely on how much the positive, specific staining compare to the non-specific background staining. The gain and exposure time chosen for the images depend on the signal to noise that the immunohistochemistry run produced. Both gain and exposure time brightens the image washing out a lot of the noise while preserving the intensity of the positively stained tissue. Generally, a gain of 1.40 and exposure time of 20 μs worked best for the data produced from the F4/80 immunohistochemical protocol.

Microscope Images:

Ten images were taken at 40x magnification on the Nikon upright microscope. The pictures are taken systematically in the areas of the tissue that positive staining appeared while avoiding regions of large empty slide space.

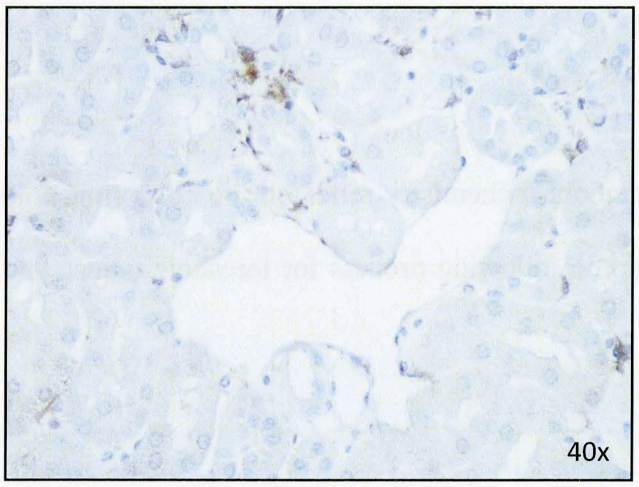


Figure 6: Example of a bad picture because there is a gaping hole in the middle of the photo and no glomerulus.

All images from one immunohistochemistry run were taken the same day to avoid any white balance variances. The pictures were not enhanced in photo software because this did not improve the analysis process. The "Auto Correct" button in Microsoft Picture Manager accomplished the same enhancement of background structures and color as the "Auto-White" enhancement in the camera program. Figure 5 displays the difference between an unedited image versus one that has been "Auto-corrected".

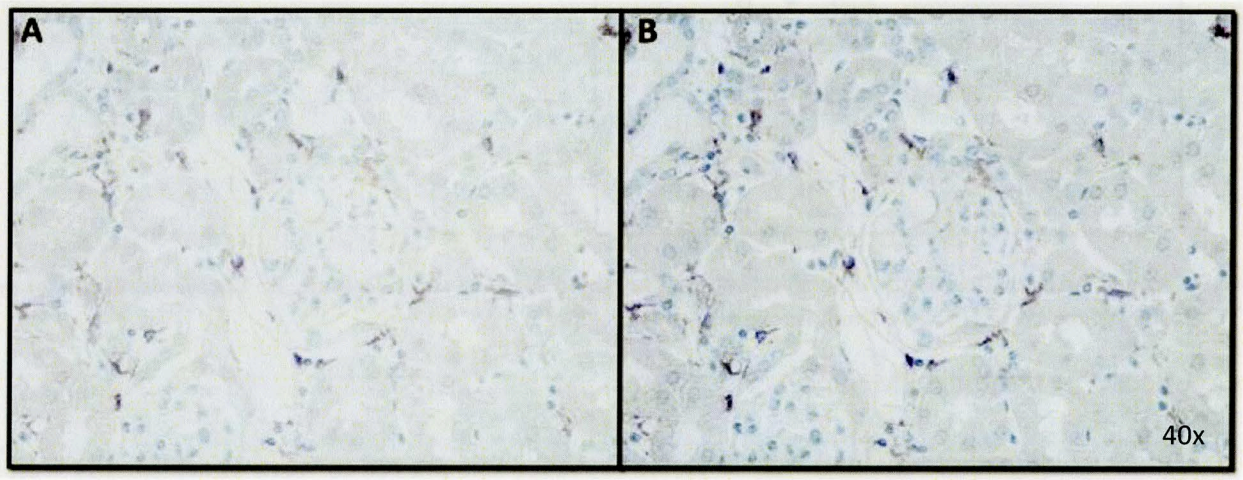


Figure 7: Difference between original image and Auto-corrected from picture software. A: Original image (exposure=20us, gain=1.40x, contrast=Anti-reflex). B: Same original image that has been "Auto-Corrected" in picture-editing software, "Auto-Correct" enhances background and makes the brown stain appear bluer.

After the images were taken and saved as either .jpeg or .tiff files they were processed and analyzed with the free NIH software, ImageJ.

ImageJ Image Analysis:

Threshold Adjustment

The basis of the quantifiable analysis process for immunohistochemistry relies on the color threshold that can be manually adjusted. After opening an image the following process for threshold adjustment was followed:

1. Click 'Image'
2. Click 'Adjust'
3. Choose 'Threshold'
    a. Threshold window dialog box appears
    b. This allows for manual adjustment of the color threshold of the picture so that the positively stained DAB color is recognized for quantitative analysis.

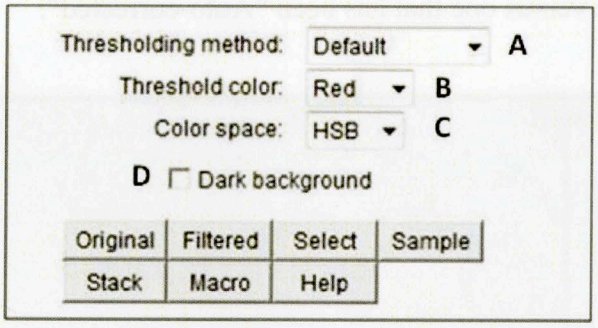


Figure 8: ImageJ Adjust Threshold Color Window. A: Threshold Method set to Default. B: Threshold Color set to Red.` C: Color Space set to HSB. D: Dark Background unchecked.

4. Choose 'Thresholding Method'
    a. The threshold method was "Default" because this proved to work exceptionally well and further complicating the process was not necessary.
5. Choose 'Threshold Color'
    a. The threshold color was "Red" because it is the most obvious color to see against the tissue images.

6. Choose 'Color Space'

    a. The color space of the threshold was Hue, Saturation, and Brightness. This color space is optimal for the DAB analysis. The Red, Green, and Blue color space was not as precise of a measurement of the brown color because instead of one value being optimized (Hue), all three values must be optimized since brown includes all of them (red, green, and blue.)

7. Uncheck 'Dark background'

    a. The 'dark background' box was unchecked so that the red threshold color is specific to the foreground brown color.

The base parameters of the color threshold window were set. The color space was adjusted to threshold the positively stained brown color in the tissue. Saturation and Brightness were changed to full capacity, the lower value set to 0 and the larger value set to 255. Saturation and Brightness were set to full capacity because Hue is the only parameter that separates the brown color, DAB stain, from the rest of the image while Brightness and Saturation increased the intensity of whatever is denoted as brown. Hue is a color wheel and adjusting the upper and lower values specifies the threshold color, brown, for the computer. The optimal lower and upper values for Hue involved trial and error but once the values are determined; each image of the immunohistochemistry run was processed with the same range for Hue. While the Hue values were adjusted, the red threshold color appeared on the tissue image and the goal was to choose settings so the red color covered the areas that have been stained brown with DAB denoting that the protein of interest was present at the specific location.

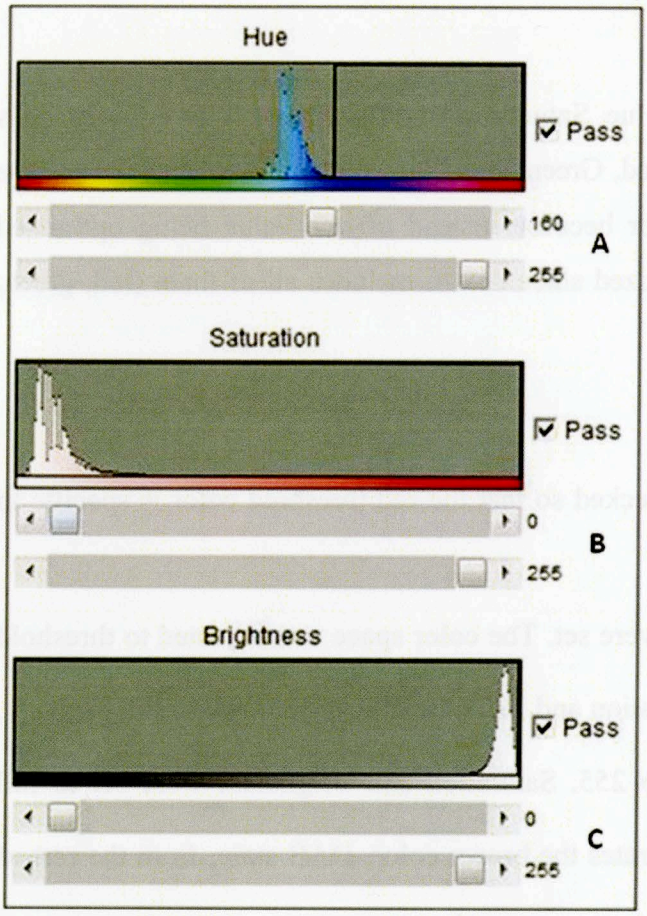


Figure 9: Adjusting HSB Values in Threshold window. A: Hue lower value will vary with immunohistochemistry runs. B: Saturation lower and upper value should always be at 0 and 255, respectively. C: Brightness lower and upper values should always be 0 and 255, respectively.

The next steps were taken to finish the color threshold process for the image:

1. Click the "Select" button
    a. Outlines in yellow all the areas that were previously highlighted in red. This step allows the user to double-check that everything inside the yellow is the positively stained DAB brown tissue.
2. Click on any of the bars for Hue, Saturation, or Brightness
    a. This caused the image to turn everything inside the yellow outline black and the background white. Once the HSB values have been fixed for the first image, with each

subsequent image opened the user will only need to click any of the bars again to turn the image black and white.

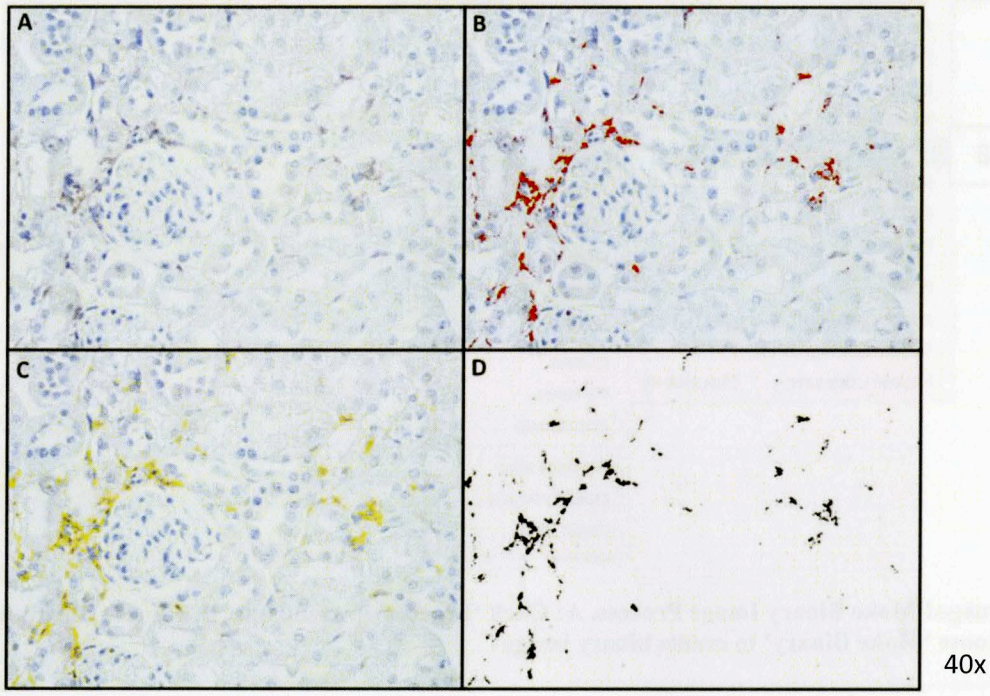


Figure 10: ImageJ Color Threshold Process. A: Original microscopy image. B: Image with Red threshold color. C: Image with Yellow section outline. D: Image prior to binary processing

Binary Image

The image was converted to binary for pixel analysis:

1. Click 'Process'
2. Choose 'Binary'
3. Click 'Make Binary'

Nothing changed superficially to the picture but the color threshold is now recognized by the software.

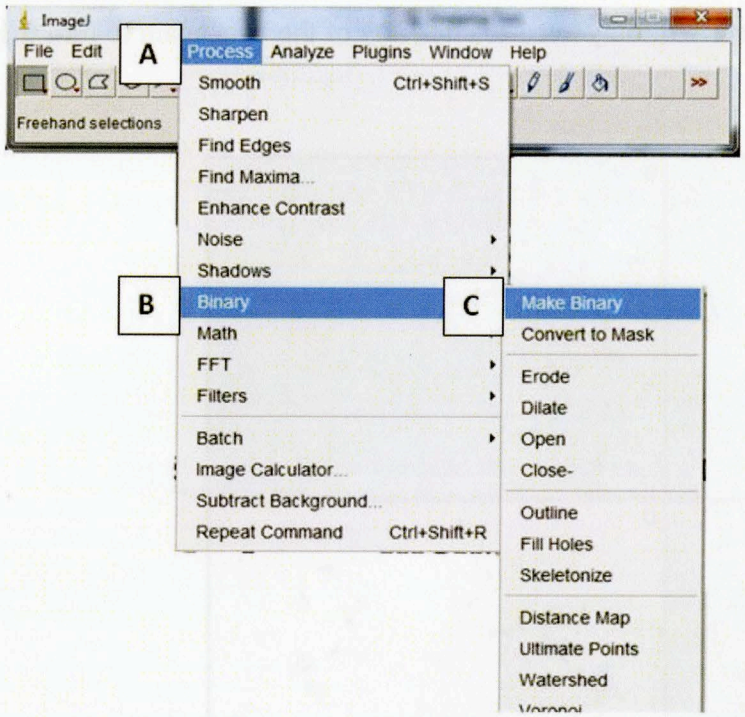


Figure 11: ImageJ Make Binary Image Process. A: Click 'Process' in menu bar. B: Choose Binary in drop-down menu. C: Choose 'Make Binary' to create binary image

Figure 12: Binary Image of Tissue Section

Filter Application

A median filter was applied to the binary image to clean the image.

1. Click 'Process'
2. Choose 'Filters'

3. Click 'Median'
    a. The 'Median Filter' dialog window opens.
4. Enter '2.0' for Radius
5. Click 'OK'

The 2.0 pixel median filter replaced each pixel with the median value from its 5 pixel neighborhood. The advantage of a median filter is that it does not introduce new values as data. The median filter only used the data that was experimentally measured to replace pixel values. A median filter retains high frequency structures while removing noise. It can reduce the amount of positive staining and it will not increase it. This critical step minimized the error associated with false positives. The filter was applied to every image.

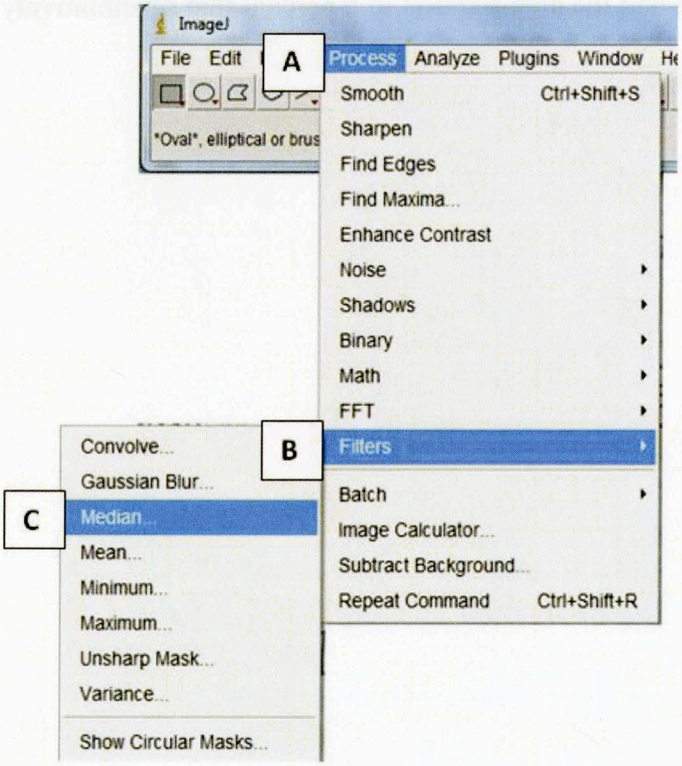


Figure 13: ImageJ Filtering Process. A: Click 'Process' in menu bar. B: Choose 'Filters' from drop-down menu. C: Choose 'Median…' for median filter application

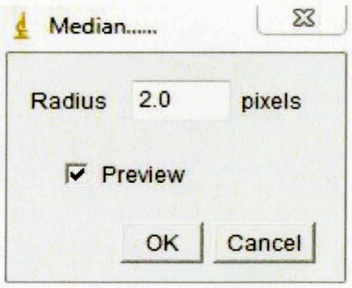


Figure 14: ImageJ Median Filter Window

Area Fraction Measurement

To measure the area fraction of positive stain in the tissue the following process was followed:

1. Click 'Analyze'
2. Choose 'Set Measurements'
    a. The 'Set Measurements' dialog window will open.
3. Uncheck 'Min & Max Gray Value' and 'Mean Gray Value'
4. Check 'Area Fraction'
5. Click OK
6. Click 'Analyze'
7. Choose 'Measure'

A new window opens displaying the total pixel area and the area fraction in a percent that quantitatively defines the amount of positive DAB stain in the image.

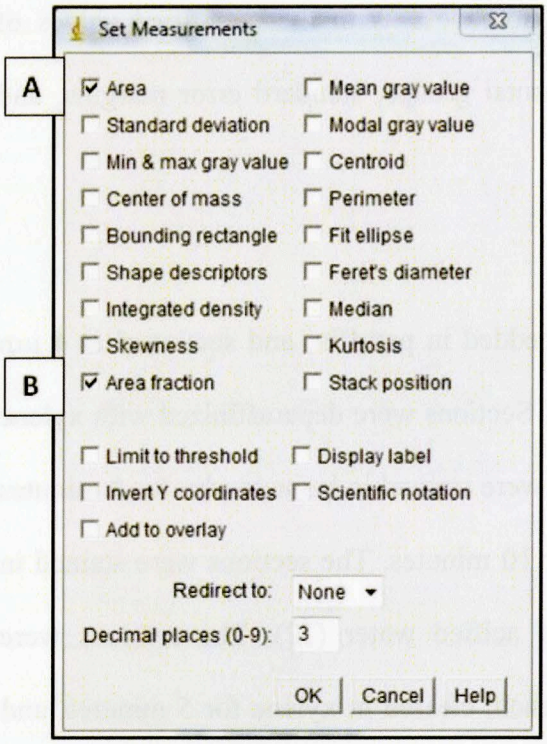


Figure 15: ImageJ Set Measurements Window. A: Check to display total area. B: Check to display area fraction of positive stain

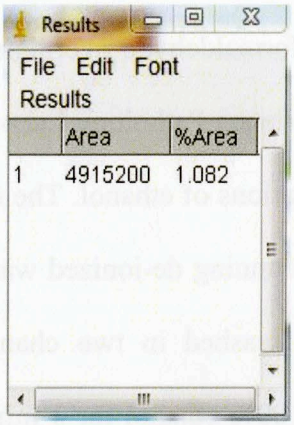


Figure 16: Measurements Results Window displaying Percent Fraction of Positive DAB Stain

Process Subsequent Images

After the HSB settings were optimized for each immunohistochemical run, subsequent images were quickly processed. The threshold window and measurements window remain open and once a new picture was opened in ImageJ any mouse click in the color threshold window triggered the threshold action on the new image. The Red color threshold and Yellow outline steps are done automatically and the black and white image appears. It was important with the first few images to complete the full color threshold process individually for each image, adjusting Hue separately and viewing the Red threshold color and Yellow outline, in order to determine the optimal lower and upper bounds for Hue. Once the range values for Hue are determined all images from the immunohistochemistry run must be processed with those same values. After the image is in black and white form the binary, filtering, and measurement steps of the process for each image were carried out. The area fraction percent measured

for each image were recorded in an Excel sheet or a lab notebook. With the quantified values of immunohistochemical stains, the differences in the experimental groups, standard error margins, and statistical analyses were calculated.

Picrosirius Red Stain Protocol:

Kidneys were fixed in 4% paraformaldehyde overnight, embedded in paraffin, and sectioned at 4 μm thickness by the University of Arizona Pathology Laboratory. Sections were deparaffinized with xylene overnight and rehydrated in gradations of ethanol. The nuclei were stained in hemaxotylin for 8 minutes and subsequently washed under running de-ionized water for 10 minutes. The sections were stained in picrosirus red for 1 hour then washed in two changes of acified water (12). The sections were dehydrated in three changes of 100% ethanol for 3 minutes each, cleared in xylene for 5 minutes, and coverslips were mounted with Permount solution. PSR stained collagen was viewed and analyzed using the Zeiss microscope and AxioVision camera program.

Zeiss Microscope Settings:

The Zeiss microscope from the Tom Doetchmann lab was used to view tissue on microscope slides that have been stained with Picrosirius Red (PSR). The PSR stain binds specifically to the birefringent substances on collagen fibrils. Under circular polarized light, collagen fibers were specifically identified by the red glow of PSR.

To start the Zeiss microscope:

1. Turn on the "Power Supply 231"
2. Turn on the microscope
3. Turn on and log into the computer

4. To configure the microscope settings to detect the birefringent collagen fiber look up Section 4.9.5.1 "Detecting Birefringence" in the Axio Imager manual

AxioVision Camera Program Settings

To view the microscope image on the computer camera software:

1. Open 'AxioVision' software
2. Click 'Live'

With the camera screen open, the camera settings were configured as followed:

1. Click 'Properties'
2. Select the 'Frame' tab
    a. Change 'Camera' to 'Fast High Definition Color'
3. Select the 'Adjust' tab
    a. Manually enter '700 μs' for 'Exposure Time'
4. Select the 'Display' tab
    a. Choose 'Exponential' for the equation of the RGB color space graph
    b. Press 'Best Fit'
    c. Adjust the 'Hue', 'Saturation', and 'Brightness' bars to approximately -0.50, 1.00. and 1.25, respectively

The 'Exponential' and 'Best Fit' equation eliminates most of the green labeled collagen and focuses on the red PSR stain on the tissue. This process and values for the camera settings worked on any PSR stained tissue because the PSR protocol is not variable; the power of the stain is equivalent as long as the protocol is over one hour.

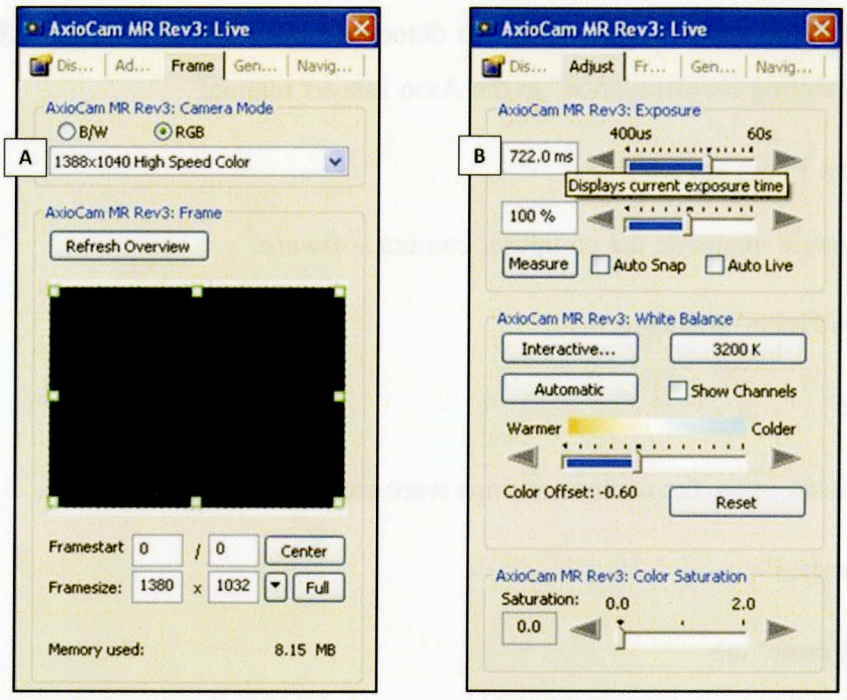


Figure 17: AxioVision Camera Settings. A: Adjust the Camera to "High Speed Color". B: Adjust exposure time to 722 µs.

Zeiss Microscope Polarizer

To preserve image resolution, images were taken at a 10x magnification. With the polarizer open no polarized light illuminated the tissue so the image on the camera was pink tissue and white slide space. When the polarizer was closed polarized light reached the tissue and the tissue was black while the PSR stained collagen fibers glow red. Two images were taken at each location; the first with the polarizer open and the second with the polarizer closed. This two-image process allowed for an exact ratio of collagen to tissue without the empty slide space.

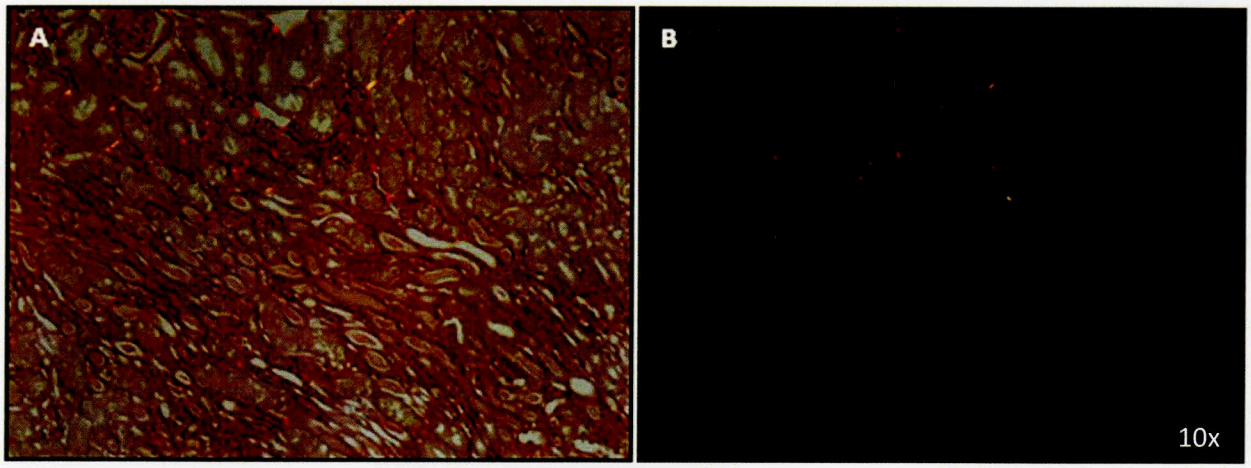


Figure 18: A: Brightened image of renal interstitial space with OPEN polarizer. B: Darkened PSR collagen of renal interstitial space with CLOSED polarizer

The pictures were taken systematically in the interstitial spaces of the tissue avoiding blood vessels and obvious consistent collagen markers like the renal medulla tip in renal tissue or ventricle circumference in cardiac tissue.

To take pictures on the Zeiss microscope:

1. Locate interstitial space with polarizer open
2. Click 'Snap'
3. 'Save' the image
4. Without moving the microscope stage, swivel the polarizer closed
    a. The birefringent substances on the PSR stained collagen should glow red on the camera screen
5. Click 'Snap'
6. 'Save' the image

With the two-image process in the exact same location the final analysis is total collagen content to total tissue area, not to total image area.

AxioVision Image Analysis:

Two .xml files were created in the AxioVision has a Program Wizard to process both images from the PSR two-image process: "EmptySlideArea.xml" and "PSRArea.xml". The two programs were used to process the brightened images and the PSR darkened images, respectively.

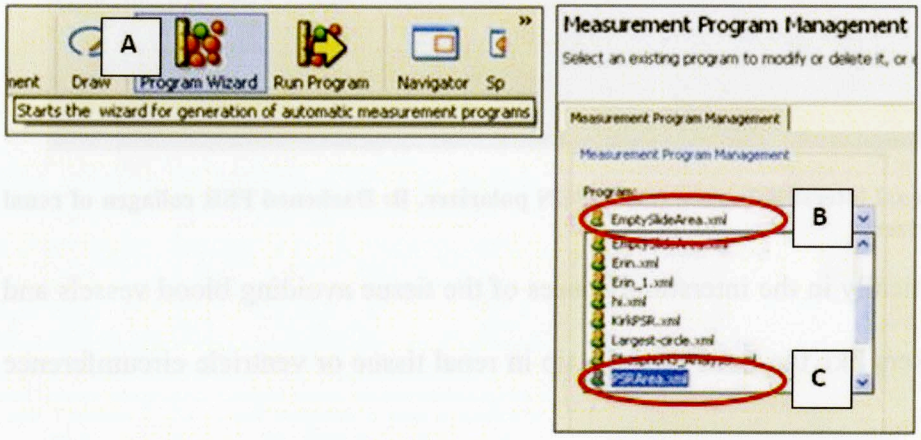


Figure 19: AxioVision Program Wizard. A: Button on Main screen. B: EmptySlideArea.xml for processing the brightened images. C: PSRArea.xml for processing the darkened PSR images.

Brightened Tissue Process

To process the brightened images taken with the polarizer open, this process was followed:

1. Open brightened image
2. Click 'Program Wizard' button
    a. The Program Wizard window will open
3. Choose 'EmptySlideArea.xml' from the drop-down menu
4. Click 'Next'
5. Use the eyedropper tool to highlight the white empty area in the tissue in the 'Phase' step
    a. The white space color can vary from slide to slide based on how open the polarizer was so the eyedropper can adjust the color threshold on each image
6. Click 'Next' in the 'Filling Holes' step

7. Click 'Finish' after approving the outlined area of empty slide space

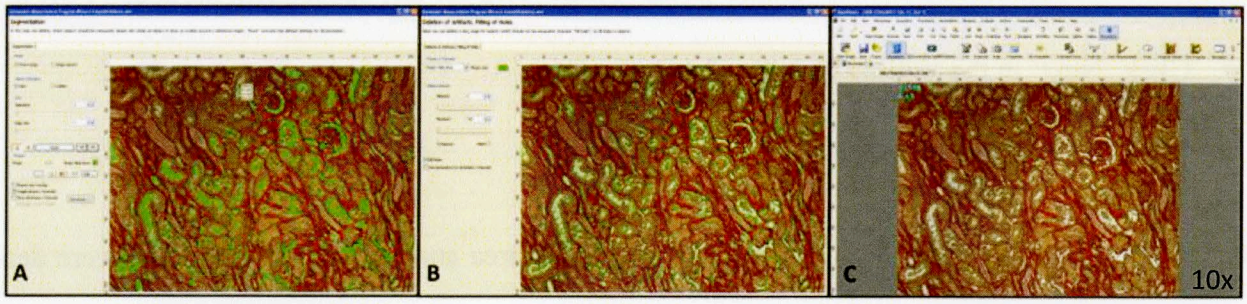


Figure 20: Empty White Space Threshold Process in AxioVision. A: Phase step where eyedropper was used to denote threshold color. B: Outline step to check threshold area. C: Final processed image with area fraction of threshold color area displayed

Once the program is finished, the final image displays the area and percent of pixels that were denoted as empty space.

Darkened Picrosirius Red Process

To process the darkened PSR images taken with the polarizer closed, this process was followed:

1. Open darkened image
2. Click 'Program Wizard' button
    a. The Program Wizard window will open
3. Choose 'PSRArea.xml' from the drop-down menu
4. Click 'Next'
5. With the first image processed, use the eyedropper tool to highlight the glowing red PSR stained collagen in the tissue in the 'Phase' step
    a. All of the PSR images should have the same red color intensity because the polarizer is in the same position for each image to get the darkest background and the camera settings never change throughout the picture-taking process. Because of this, the threshold color set by the eyedropper in the first darkened PSR image is capable of thresholding each image in the session.

b. To reduce error in the analysis, the same threshold is applied to each darkened PSR image so do not click 'Reset' in the 'Phase' step for any subsequent darkened PSR image

6. Click 'Next' in the 'Filling Holes' step

7. Click 'Finish' after approving

Once the program is finished, the final image displays the area and percent of pixels that were denoted as interstitial collagen within the tissue.

Area Percent Measurement

Figure 15 illustrates the steps that the programs follow in order to quantify the threshold areas:

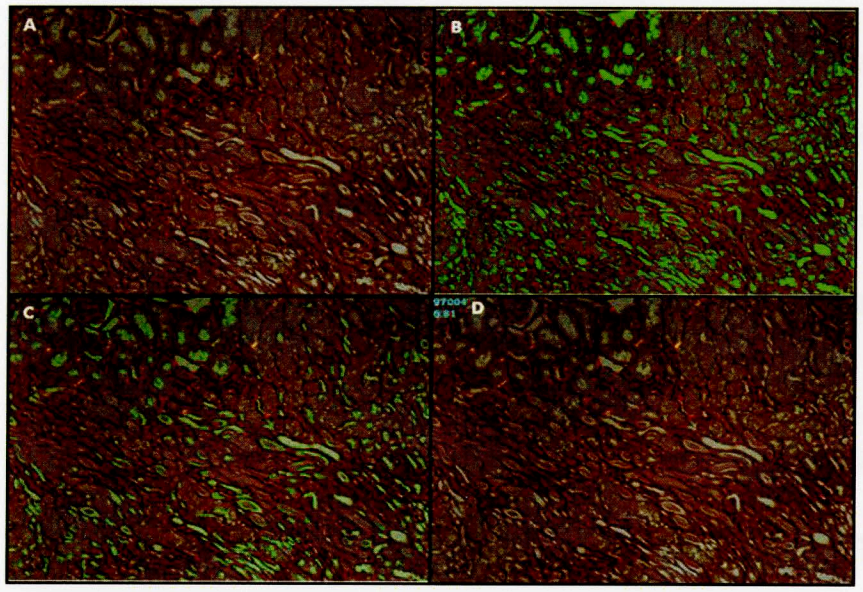
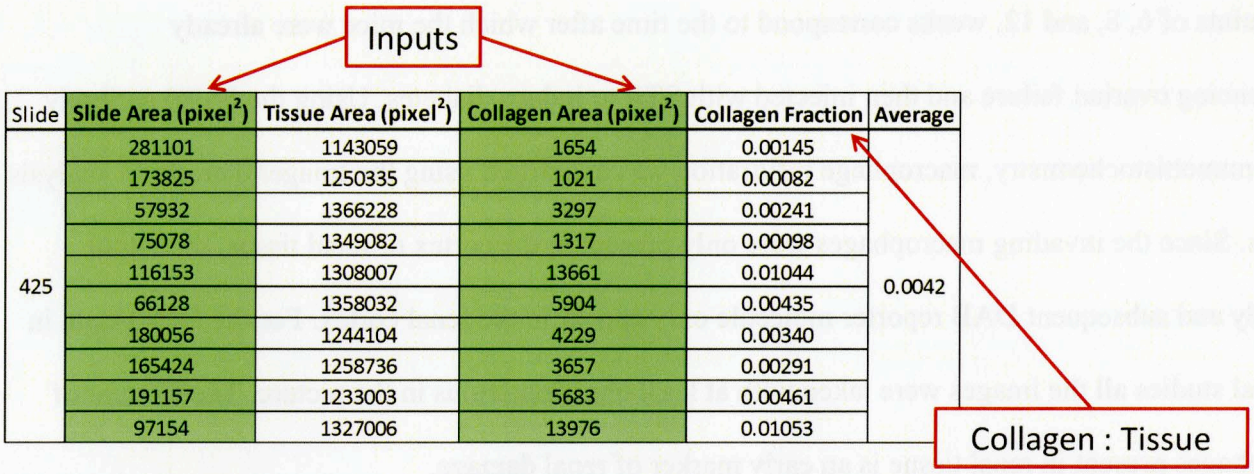


Figure 21: AxioVision Threshold Process with EmptySlideSpace.xml Program. A: Original AxioVision PSR brightened image. B: AxioVision Phase step with eye-dropper denoting empty slide space. C: AxioVision outline step checking empty slide space. D: Finished AxioVision PSR brightened image with pixel area and percent of empty space

Table 1 shows an Excel sheet that the user can use to record and analyze the data:



Slide	Slide Area (pixel^2)	Tissue Area (pixel^2)	Collagen Area (pixel^2)	Collagen Fraction	Average
425	281101	1143059	1654	0.00145	0.0042
	173825	1250335	1021	0.00082	
	57932	1366228	3297	0.00241	
	75078	1349082	1317	0.00098	
	116153	1308007	13661	0.01044	
	66128	1358032	5904	0.00435	
	180056	1244104	4229	0.00340	
	165424	1258736	3657	0.00291	
	191157	1233003	5683	0.00461	
	97154	1327006	13976	0.01053	

Table 1: Example Excel table for PSR Processing

After processing all of the images of each slide in the picrosirius red stain protocol and recording the area fraction values, the differences in the experimental groups, standard error margins, and statistical analyses were calculated.

Statistics:

Data were analyzed using unpaired Student's *t-test* to identify differences between groups. In all tests, $P < 0.05$ was considered significant. Results are presented as mean ± SD or SE. In the appendices are detailed tables with raw data and statistical results. For some studies, more appropriate analyses are included like a One-Way or Two-Way ANOVA.

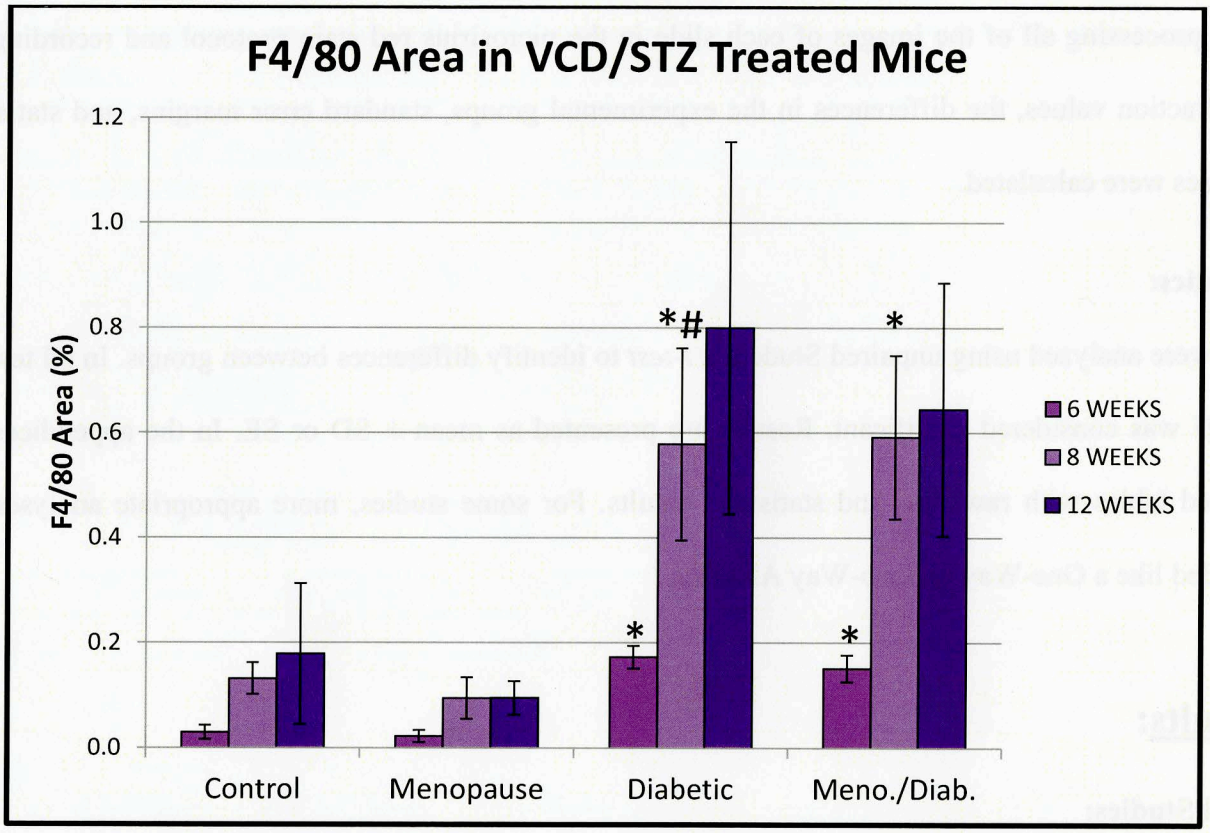
Results:

Renal Studies:

Diabetic Kidney Disease in VCD Model of Menopause

This experiment studied groups of mice that were dosed with VCD and STZ or both to cause menopausal, diabetic, and menopausal diabetic mice, respectively. The mouse model was created to

study the effect of menopause on the development of diabetes and diabetic kidney damage (11). The 3 time-points of 6, 8, and 12, weeks correspond to the time after which the mice were already experiencing ovarian failure and then injected with STZ to induce diabetes. Using the F4/80 antibody and immunohistochemistry, macrophage infiltration was quantified using the ImageJ Threshold analysis process. Since the invading macrophages were only present in the cortex of renal tissue, the F4/80 antibody and subsequent DAB reporter molecule only appear in the renal cortex. For the F4/80 stain in the renal studies all the images were taken with at least one glomerulus in the picture. The amount of macrophages present in renal tissue is an early marker of renal damage.



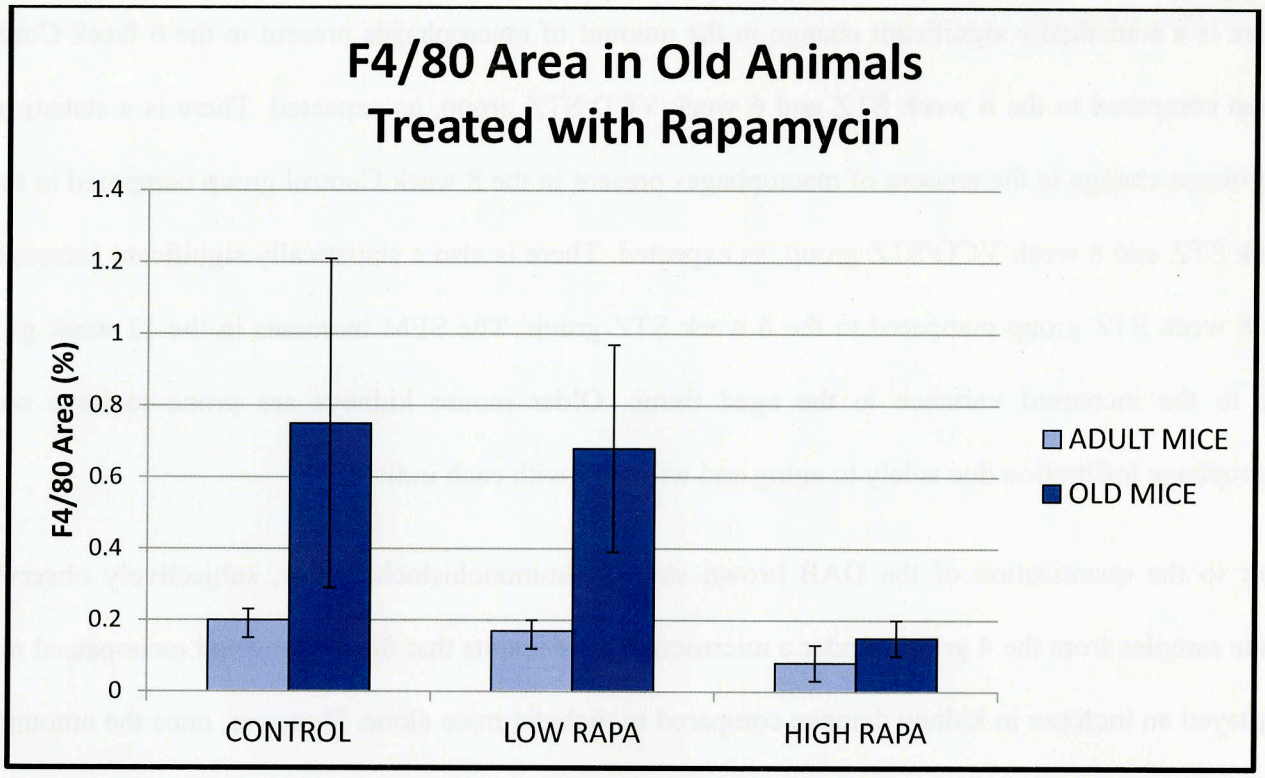
Graph 1: F4/80 Area Fraction in MK-020 Study. The data shown are the percentage of positively stained brown tissue marking the F4/80 antigen present on invading macrophages. There is a significant difference ($p<0.05$) of the 6 week VCD/STZ mice compared to 6 week Control and a significant difference ($p<0.05$) of the 8 week VCD/STZ mice compared to 8 week Control. There is also a significant difference in the Diabetic group between the 6 week and 8 week mice denoted with the # sign ($p<0.05$). There is no difference in macrophage infiltration between the Diabetic and Menopausal Diabetic groups.

There is a statistically significant change in the amount of macrophages present in the 6 week Control group compared to the 6 week STZ and 6 week VCD/STZ group, as expected. There is a statistically significant change in the amount of macrophages present in the 8 week Control group compared to the 8 week STZ and 8 week VCD/STZ group, as expected. There is also a statistically significant increase in the 8 week STZ group compared to the 6 week STZ group. The SEM increases in the 12 week group due to the increased variance in the aged tissue. Older mouse kidneys are prone to have some macrophage infiltration due solely to aging and will vary with each individual.

Prior to the quantization of the DAB brown stain in immunohistochemistry, subjectively observing tissue samples from the 4 groups under a microscope gave results that the diabetic and menopausal mice displayed an increase in kidney damage compared to diabetic mice alone. However, once the amount of F4/80 protein was given a number value through the ImageJ Threshold process the results show that there is no significant difference in macrophage infiltration between menopausal diabetic mice versus diabetic mice alone for 6 week and 8 week groups, p values of 0.5434 and 0.9570, respectively.

Rapamycin Treated Old Animals with Chronic Kidney Disease

The hypothesis that rapamycin (RAPA) can be used as a drug to counteract macrophage infiltration in old mice (>18 months old) was correct. RAPA is an immunosuppressant drug. The adult and old mice were roughly 12 weeks old and 18 months old, respectively. This study dosed adult mice and old mice with a low dose of RAPA and a high dose of RAPA, 75 μg and 2.5 mg, respectively. Macrophage infiltration was quantified using the F4/80 antibody immunohistochemistry protocol and ImageJ Threshold process.



Graph 2: F4/80 Area Fraction in JRA-204 Study. No statistical significance amongst the Old Control and Old High RAPA groups but the low n of 3 does indicate a trend that higher doses of RAPA can rescue the inflamed disease state of aged mice.

There is not a statistically significant difference in Old mice given the high RAPA dose compared to Old Control mice. However, it is clear from the quantified data that there is distinct damage that occurs to kidneys as a result of age. The SEM bars are large in the Old mice groups due to the variances in aged kidneys. The data involves a low n (n=3) so although the trend is that high dose of RAPA rescues the disease state in the aged mice, the results are not statistically significant.

Collagen Deposition in Rapamycin Treated Old Animals with Chronic Kidney Disease

Collagen deposition in renal tissue is indicative of kidney fibrosis – the thickening and hardening of the kidney leading to kidney failure due to the loss of filtration strength (9). The picrosirius red stain was used to tag the collagen fibers and circularly polarized light was used to view the collagen in renal tissue (19). In renal tissues the collagen present in the interstitial space is what is of interest because collagen in the interstitial space is indicative of kidney damage and disease. There are sources of collagen that are

consistent in all kidneys such as the collagen surrounding blood vessels and the inner medulla tip so when taking pictures the user needs to avoid these tissue artifacts and focus on the interstitial space of the renal tissue.

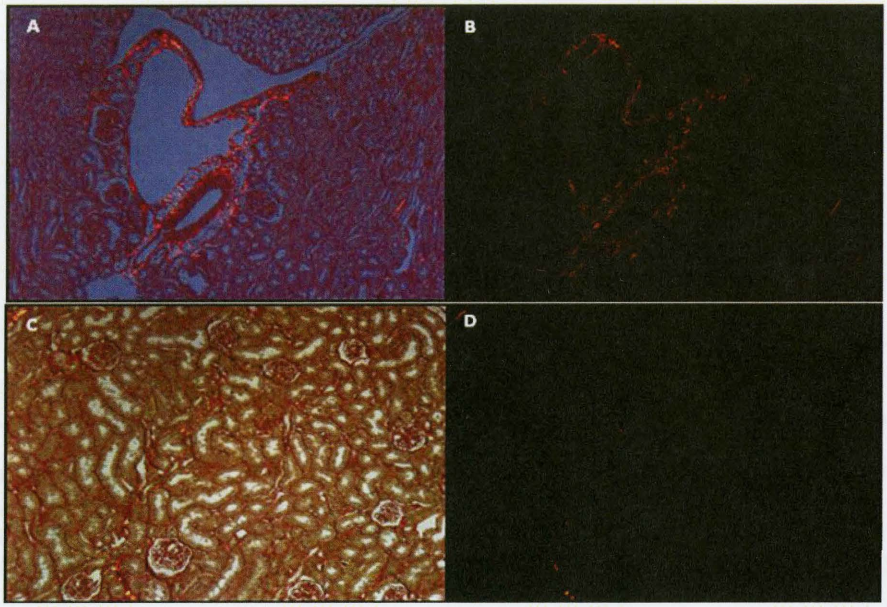
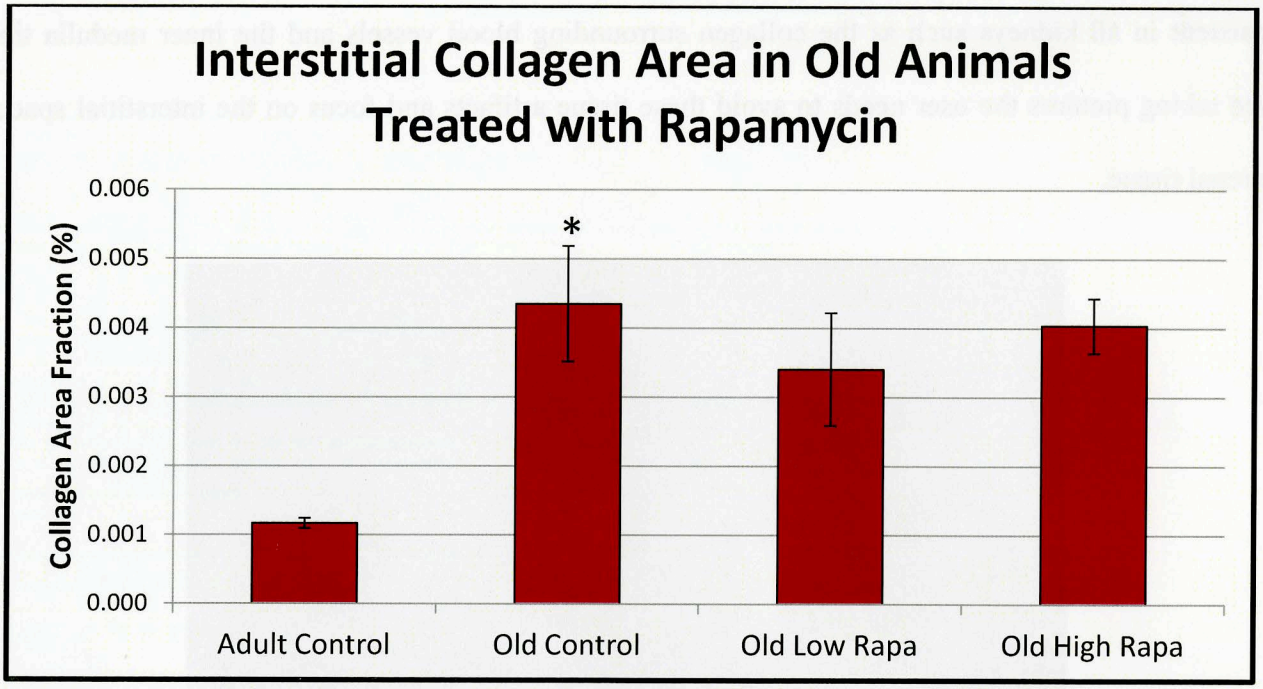


Figure 22: A: Brightened image of renal blood vessel and medullary cap. B: Darkened PSR image of Image A highlighting collagen content. C: Brightened image of renal interstitial cortex. D: Darkened PSR image of Image C highlighting collagen content.

It is very important to take unbiased, systematic pictures of the interstitial space of the tissue; for renal tissue, the interstitial collagen is not localized to a certain area so images need to be taken from the cortex, outer medulla, and inner medulla. For cardiac tissue, the user needs to avoid the consistent collagen present surrounding the atrias and ventricles and again, focus on the interstitial space.

The AxioVision programs EmptySlideArea.xml and PSRArea.xml were used to quantify the amount of collagen in the tissue.



Graph 3: Interstitial Collagen Area Fraction in JRA-204 Study. The data shown are the percentage of interstitial collagen present in renal tissue of old mice (>18 months old) given a low and high dose of RAPA and young (~12 weeks old) Control mice. There is a significant difference in the presence of collagen deposition of Old Control mice compared to Young Control mice, p=0.0355. RAPA has no significant effect on collagen deposition in renal tissue of Old mice.

This study conclusively shows that there is a significant difference in collagen deposition between the Young and Old Control groups with a p-value of 0.0355. Rapamycin treated old mice do not show a decrease in collagen present, thus rapamycin is not a sufficient treatment for reducing collagen deposition in old kidneys affected with chronic kidney disease. As seen in the previous studies, an intrinsic standard error is present when analyzing the kidneys of older mice.

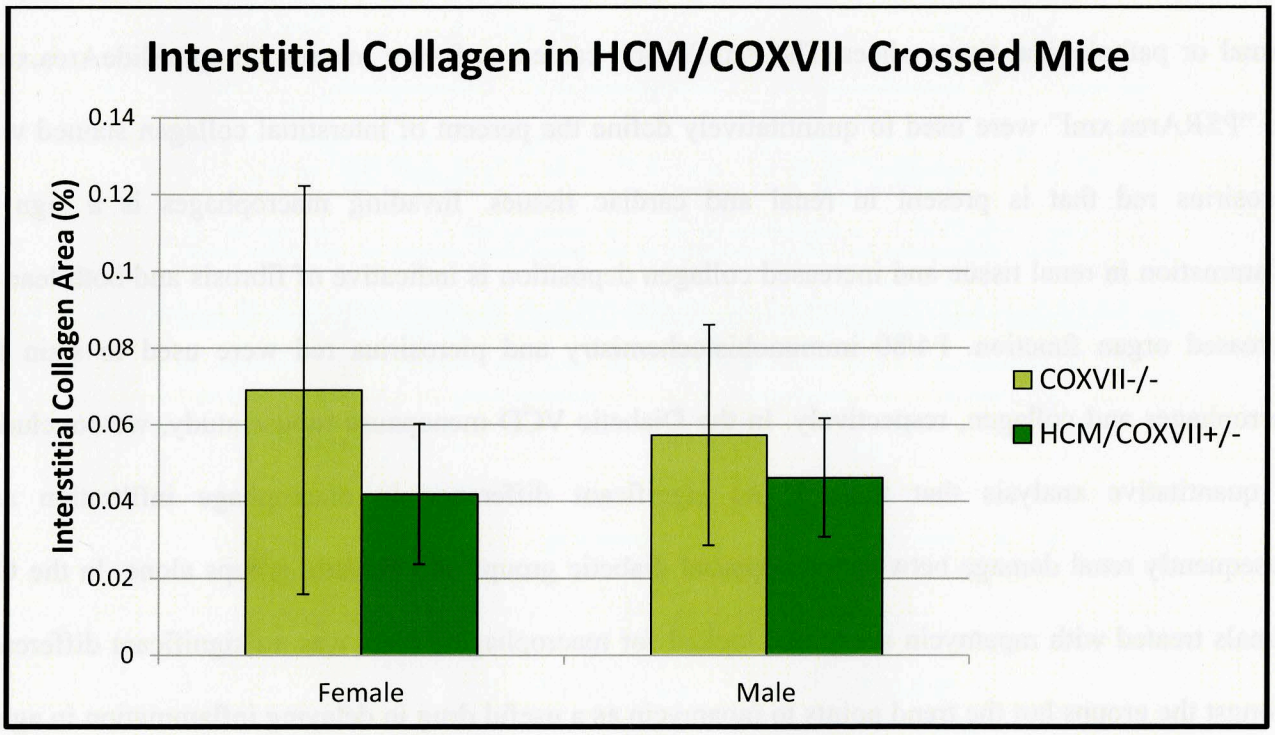
Cardiac Study:

Cardiac HCM/COXVII KO Crossed Mice

In this cardiac study, the transgenic mouse line with a R403Q change in the alpha-myosin heavy chain of cardiac muscle develop hypertrophic cardiomyopathy (HCM) around 12 weeks old and are crossed with the knock-out mouse model that deletes the COX7a1 gene (COX7a1 KO), the major cardiac isoform, that cause the mice to develop dilated cardiomyopathy around 6 weeks old. It was known that the crossed mice (HCM/COX7a1 KO) develop larger hearts than the COX7a1 KO mice alone. The

question is whether increased collagen deposition correlates with the enlarged hearts. Using the Picrosirius red stain, circularly polarized light (25) and the AxioVision Color Threshold Analysis the amount of collagen deposition in the interstitial tissue of the cardiac tissue was quantified.

The quantified data illustrates that there is no correlation between the hypertrophic cardiomyopathy crossed with COXV7a1 knockout mice and changes in collagen deposition. There was a low "n" associated with this study but another reason for the null correlation is that the crossed mutant mice (HCM/COX7a1 KO) may not develop the hypertrophy at all and instead progress straight to dilated cardiomyopathy like the COX7a1 knock-outs mice.



Graph 4: Average Cardiac Interstitial Collagen Area in HCM/COX7a1 KO vs COX7a1. There is no statistical significance of differences amongst the groups.

Discussion:

Conclusion:

The current standard for data analysis from immunohistochemistry is to qualitatively make comparisons on the amount of positive staining seen amongst the experimental groups in a study (4). There is a need for a quantitative, inexpensive, and easy process to assign value to the amount of positive staining seen in tissue so that data can be more conclusive. The free NIH downloadable software, ImageJ, used a color threshold process to quantitatively analyze immunohistochemistry staining using DAB as the reporter molecule with the methods clearly defined. The quantification of immunostaining by means of digital image analysis allows the characterization of protein expression revealed by immunohistochemistry on normal or pathological tissue slices. The AxioVision camera software macros "EmptySlideArea.xml" and "PSRArea.xml" were used to quantitatively define the percent of interstitial collagen stained with picrosirius red that is present in renal and cardiac tissues. Invading macrophages is a sign of inflammation in renal tissue and increased collagen deposition is indicative of fibrosis and both lead to decreased organ function. F4/80 immunohistochemistry and picrosirius red were used to stain for macrophages and collagen, respectively. In the Diabetic VCD-menopause mouse study, we concluded by quantitative analysis that there is no significant difference in macrophage infiltration and consequently renal damage between menopausal diabetic groups and diabetic groups alone. In the Old animals treated with rapamycin study that looked for macrophages, there was no significant difference amongst the groups but the trend points to rapamycin as a useful drug in delaying inflammation in aging mice kidneys. Looking at collagen deposition in the same renal study there was a significant difference between the Adult control and Old control groups illustrating the known fact that collagen deposition is an effect of an aging kidney; we can also conclude that rapamycin is not a useful drug in delaying collagen deposition. In the hypertrophic cardiomyopathic mice, no significant correlation between the

knock-out COXVII gene and collagen deposition was found. The color threshold analysis is an effective, non-subjective, and reliable method for quantifying protein expression in immunohistochemical images.

Notes and Improvements for Future Work:

Immunohistochemistry

When this project is further developed and applied to other animal studies a robust improvement would be to stain several sections of tissue from the same animal – a practice not often done with immunohistochemistry. The different sections from the same animal would be stained in separate immunohistochemical runs and would be more representative of the animal. A caution with this improved method would be that if the protein of interest is located in a specific region of the organ then some slices of tissue may be void completely of the region of localized protein. For example, the AQP-1 antibody stains for the Aquaporin-1 protein that is only present in the collecting ducts of the kidney located in the inner medulla. In mice, the medulla becomes easily detached from the rest of the kidney so many tissue sections lack the tip of the medulla. With this in mind, quantifying protein expression throughout the animal's organ will reduce biased error and provide a more complete, inclusive view of the amount of protein present in the tissue.

Color Space for Threshold Analysis

In the studies that used diaminobenzine (DAB) immunohistochemistry, the Hue, Saturation, Brightness (HSB) color space was found to work best because of the decreased number of parameters to optimize compared to Red, Green, Blue (RGB) color space. However, before the Picrosirius red (PSR) stained sections were quantified using AxioVision the images were processed in ImageJ with the same methods as described for DAB immunohistochemistry. The difference was that ImageJ was used to create an RGB Stack when the image is split into 3 channels: red, green, and blue. The red channel image was used to define a threshold and quantify the amount of interstitial collagen present. This process in

ImageJ yielded analogous results to when the PSR images were processed in AxioVision. The take-home message is that dependant on the type of immunohistochemical stain used the optimal color space to threshold the positively stained areas may change.

Other types of stain that was experimented with throughout this work included Periodic Acid Schiff (PAS), Masson's Tri-chrome, and hematoxylin and eosin (H&E). These stains do not require polarized light to view the protein or structure of interest. The following image illustrates the vibrant colors of PAS, Tri-chrome, and H&E:

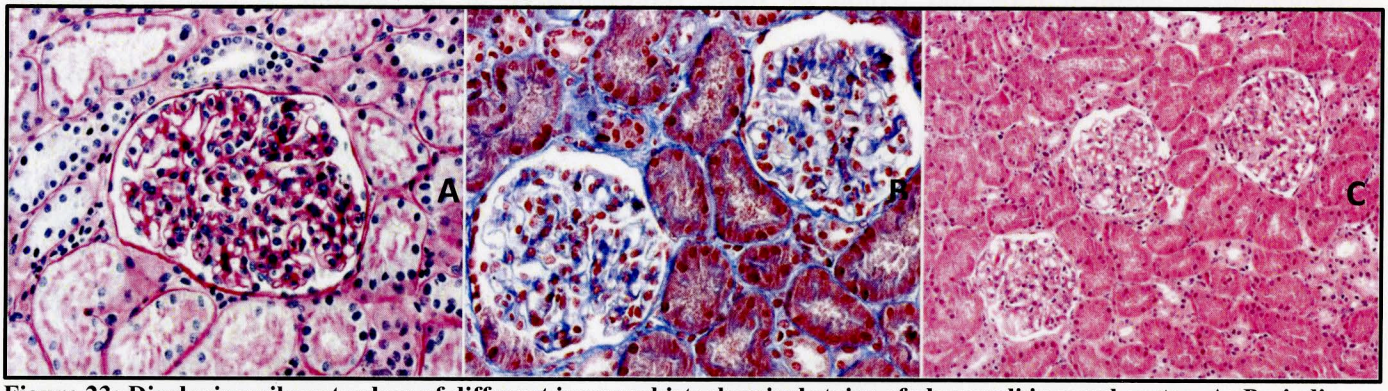


Figure 23: Displaying vibrant colors of different immunohistochemical stains of glomeruli in renal cortex. A: Periodic Acid Schiff (PAS) magnified 200x. B: Masson's Tri-Chrome magnified 200x. C: Hematoxlin and Eosin (H&E) magnified 100x.

These stains were more difficult to threshold than the diaminobenzine or picrosirius red since the colors in each stain are nearly monochromatic. A recommended improvement from the committee during the defense of this thesis is to use the RGB color space instead of HSB for every immunohistochemical stain.

Since Hue is a multifaceted cone comprised of the red, green, and blue colors with the added parameters of lightness and saturation, the RGB color space is more definitive and accurate of the colors present in an image.

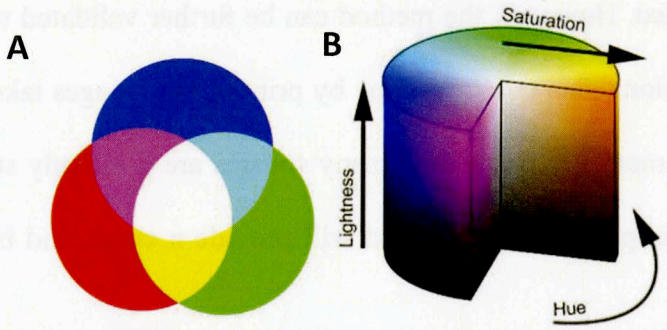


Figure 24: Image describing the RGB and HSB color spaces. A: The color wheel defined by Red, Green, and Blue. B: Hue defined by 3 values of color (red, green, and blue) while lightness and saturation help determine strength and brightness of the defined color.

Using the RGB space with the brightly colored immunohistochemical stains could allow for quantitative analysis. Even with the DAB brown stain, if the RGB color space was used to extract the raw color data from the original image the brown color could be defined against the rest of the tissue. That brings us to the question of how to standardize the background or non-specific staining that inevitably varies with each immunohistochemical run.

This could be done with a program or written code in Matlab or other software that would take a snapshot of the background color of each stained section and effectively apply a white balance filter to make it so that the background of every slide from every immunohistochemical run is the same. This would allow for the color parameters (Hue if using HSB or red, green, and blue if using RGB) to remain constant once the optimal values have been determined. This improved process would be more robust and decrease human error involved in the immunohistochemistry, picture-taking, and quantitative analysis procedures.

Lastly, the quantitative analysis described in this work is considered objective and accurate because of the effort to decrease error and if there is inherent error, then to standardize it. The color threshold process is validated by comparing the trends and results from the renal quantitative analysis to blood glucose levels, albumin urea ratios, and urine osmolality of the animal subjects. Since the results of the quantitative analysis reflected what was happening physiologically to the animals then the color

threshold process is validated. However, the method can be further validated with a manual standard of quantifying protein expression. This could be done by printing the images taken of a tissue section and using a transparent grid to manually count how many squares are positively stained to the total area of the grid. The standard is simple and minimal and will provide a check and balance for the computer-aided threshold analysis.

Statistics

The graphs in the Results section reflect statistical significance as determined by a Student's *t-test*. However, if the work were to be reevaluated, more conservative and inclusive statistical analyses would be completed.

The study that involved VCD/STZ treated mice that became menopausal and/or diabetic requires a Two-Way ANOVA (Analysis of Variance) because there are two factors involved in the experiment: experimental group and time-point. The *t-test* and Two-Way ANOVA data can be found in the Appendix for this study. The study analyzing old animals with chronic kidney disease treated with Rapamycin would require a One-Way ANOVA to look for statistically significant interactions. Originally, the *t-test* resulted in no significant differences when F4/80 was used to stain for macrophages so an ANOVA was not performed. The *t-test* in the same study that looked for interstitial collagen did result in statistical significance so an ANOVA on the Ranks analysis was performed to check if the difference remained significant with a more conservative analysis. These data can be found in the Appendix. The cardiac study showed zero correlation with a *t-test* so another analysis was not performed; this data is available in the Appendix. As more studies are quantified using the color threshold analysis more appropriate and robust statistical analyses will be performed with the data.

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Appendix:

Statistics for Menopausal Diabetic Mice Study:

Student's Unpaired t-test Statistical Analysis for F4/80 Area:

Although a t-test is unsuitable for this data, it was the original statistical analysis used. The following tables display the values to compare experimental groups within the time-points and to compare time-points within the experimental groups. Differences were considered statistically significant if $p<0.05$.

* The p-values in **bold** denote statistical significance ($p<0.05$).

6 WEEKS							
Student's Unpaired t-test							
CONTROL	**STZ**		**CONTROL**	**VCD/STZ**		**STZ**	**VCD/STZ**
0.006	0.1494		0.006	0.1891		0.1494	0.1891
0.049	0.1784		0.049	0.183		0.1784	0.183
0.056	0.133		0.056	0.1552		0.133	0.1552
0.008	0.2308		0.008	0.0777		0.2308	0.0777
***p* =**	**0.0013**		***p* =**	**0.0055**		*p* =	0.5434

8 WEEKS							
Student's Unpaired t-test							
CONTROL	**STZ**		**CONTROL**	**VCD/STZ**		**STZ**	**VCD/STZ**
0.0609	0.3951		0.0609	0.2786		0.3951	0.2786
0.1047	0.1508		0.1047	0.3839		0.1508	0.3839
0.1724	0.8998		0.1724	0.9343		0.8998	0.9343
0.1897	0.8665		0.1897	0.7696		0.8665	0.7696
p =	0.0531		***p* =**	**0.0272**		*p* =	0.9570

Student's Unpaired t-test							
CONTROL	**STZ**		**CONTROL**	**VCD/STZ**		**STZ**	**VCD/STZ**
0.037	0.6761		0.037	0.8775		0.6761	0.8775
0.58	0.4295		0.58	0.3188		0.4295	0.3188
0.0641	0.261		0.0641	0.1742		0.261	0.1742
0.0338	1.8325		0.0338	1.2073		1.8325	1.2073
p =	0.1525		*p* =	0.1424		*p* =	0.7296

CONTROL							
Student's Unpaired t-test							
6 WKS	**8 WKS**		**6 WKS**	**12 WKS**		**8 WKS**	**12 WKS**
0.006	0.0609		0.006	0.037		0.0609	0.037
0.049	0.1047		0.049	0.58		0.1047	0.58
0.056	0.1724		0.056	0.0641		0.1724	0.0641
0.008	0.1897		0.008	0.0338		0.1897	0.0338
***p* =**	**0.0206**		*p* =	0.3107		*p* =	0.7447

STZ						
Student's Unpaired t-test						
6 WKS	**8 WKS**		**6 WKS**	**12 WKS**	**8 WKS**	**12 WKS**
0.1494	0.3951		0.1494	0.6761	0.3951	0.6761
0.1784	0.1508		0.1784	0.42952	0.1508	0.42952
0.133	0.8998		0.133	0.261	0.8998	0.261
0.2308	0.8665		0.2308	1.8325	0.8665	1.8325
p =	0.0704		*p* =	0.1281	*p* =	0.5986

STZ/VCD						
Student's Unpaired t-test						
6 WKS	**8 WKS**		**6 WKS**	**12 WKS**	**8 WKS**	**12 WKS**
0.1891	0.2786		0.1891	0.8775	0.2786	0.8775
0.183	0.3839		0.183	0.3188	0.3839	0.3188
0.1552	0.9343		0.1552	0.1742	0.9343	0.1742
0.0777	0.7696		0.0777	1.2073	0.7696	1.2073
***p* =**	**0.0314**		*p* =	0.0883	*p* =	0.8600

Two-Way ANOVA with Tukey Post-Hoc Statistical Analysis for F4/80 Area:
After the defense discussion about statistics, I ran a Two-Way ANOVA using SigmaStat and chose the Pairwise Multiple Comparison Tukey procedure for the Post-Hoc test to look for significant interactions.

The values highlighted in yellow denote statistical significance ($p<0.05$). This analysis is more appropriate for these data because it accounts for the 2 different factors, experimental group and time-point. Some of the same comparisons that were found to be statistically significant using a *t-test* remain significant with the Two-Way ANOVA but overall, this analysis is more conservative.

Two Way Analysis of Variance

Data source: Data 1 in Notebook

Balanced Design

Dependent Variable: F4/80 AREA in VCD/STZ Treated Mice

Normality Test: Failed (P = <0.001)

Equal Variance Test: Failed (P = 0.008)

Source of Variation	DF	SS	MS	F	P
WEEK	2	0.975	0.487	5.529	0.008
GROUP	3	1.941	0.647	7.340	<0.001
WEEK x GROUP	6	0.486	0.0811	0.920	0.492
Residual	36	3.173	0.0881		
Total	47	6.574	0.140		

The difference in the mean values among the different levels of WEEK is greater than would be expected by chance after allowing for effects of differences in GROUP. There is a statistically significant difference (P = 0.008). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of GROUP is greater than would be expected by chance after allowing for effects of differences in WEEK. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of WEEK does not depend on what level of GROUP is present. There is not a statistically significant interaction between WEEK and GROUP. (P = 0.492)

Power of performed test with alpha = 0.0500: for WEEK: 0.740

Power of performed test with alpha = 0.0500: for GROUP: 0.957

Power of performed test with alpha = 0.0500: for WEEK x GROUP: 0.0500

Least square means for WEEK:

Group	**Mean**
6 Weeks	0.0942
8 Weeks	0.345
12 Weeks	0.430

Std Err of LS Mean = 0.0742

Least square means for **GROUP**:

Group	**Mean**
CON	0.101
VCD	0.0790
STZ	0.517
VCD/STZ	0.463

Standard Error of LS Mean = 0.0857

Least square means for **WEEK x GROUP**:

Group	**Mean**
6 Weeks x CON	0.0297
6 Weeks x VCD	0.0230
6 Weeks x STZ	0.173
6 Weeks x VCD/STZ	0.151
8 Weeks x CON	0.0930
8 Weeks x VCD	0.119
8 Weeks x STZ	0.578
8 Weeks x VCD/STZ	0.592
12 Weeks x CON	0.179
12 Weeks x VCD	0.0953
12 Weeks x STZ	0.800
12 Weeks x VCD/STZ	0.645

Standard Error of LS Mean = 0.148

ALL PAIRWISE MULTIPLE COMPARISON PROCEDURES (**Tukey Test**):

Comparisons for factor: **WEEK**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
12 Weeks vs. 6 Weeks	0.335	3	4.520	0.008	Yes
12 Weeks vs. 8 Weeks	0.0842	3	1.134	0.704	No
8 Weeks vs. 6 Weeks	0.251	3	3.385	0.056	No

Comparisons for factor: **GROUP**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
STZ vs. VCD	0.438	4	5.111	0.005	Yes
STZ vs. CON	0.416	4	4.860	0.008	Yes
STZ vs. VCD/STZ	0.0545	4	0.636	0.969	No
VCD/STZ vs. VCD	0.384	4	4.475	0.016	Yes
VCD/STZ vs. CON	0.362	4	4.224	0.025	Yes
CON vs. VCD	0.0215	4	0.251	0.998	No

Comparisons for factor: **GROUP within 6**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
STZ vs. VCD	0.150	4	1.009	0.891	No
STZ vs. CON	0.143	4	0.963	0.904	Do Not Test
STZ vs. VCD/STZ	0.0215	4	0.145	1.000	Do Not Test
VCD/STZ vs. VCD	0.128	4	0.864	0.928	Do Not Test
VCD/STZ vs. CON	0.122	4	0.819	0.938	Do Not Test
CON vs. VCD	0.00675	4	0.0455	1.000	Do Not Test

Comparisons for factor: **GROUP within 8**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
VCD/STZ vs. CON	0.499	4	3.360	0.100	No
VCD/STZ vs. VCD	0.473	4	3.187	0.128	Do Not Test
VCD/STZ vs. STZ	0.0135	4	0.0910	1.000	Do Not Test
STZ vs. CON	0.485	4	3.269	0.114	Do Not Test
STZ vs. VCD	0.460	4	3.096	0.146	Do Not Test
VCD vs. CON	0.0257	4	0.173	0.999	Do Not Test

Comparisons for factor: **GROUP within 12**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
STZ vs. VCD	0.705	4	4.748	0.010	Yes
STZ vs. CON	0.621	4	4.185	0.027	Yes
STZ vs. VCD/STZ	0.155	4	1.048	0.880	No
VCD/STZ vs. VCD	0.549	4	3.700	0.060	No
VCD/STZ vs. CON	0.466	4	3.138	0.138	Do Not Test
CON vs. VCD	0.0835	4	0.563	0.978	Do Not Test

Comparisons for factor: **WEEK within CON**

Comparison	Diff of Means	p	q	P	P<0.05
12 Weeks vs. 6 Weeks	0.149	3	1.004	0.759	No
12 Weeks vs. 8 Weeks	0.0858	3	0.578	0.912	Do Not Test
8 Weeks vs. 6 Weeks	0.0633	3	0.426	0.951	Do Not Test

Comparisons for factor: **WEEK within VCD**

Comparison	Diff of Means	p	q	P	P<0.05
8 Weeks vs. 6 Weeks	0.0958	3	0.645	0.892	No
8 Weeks vs. 12 Weeks	0.0235	3	0.158	0.993	Do Not Test
12 Weeks vs. 6 Weeks	0.0723	3	0.487	0.937	Do Not Test

Comparisons for factor: **WEEK within STZ**

Comparison	Diff of Means	p	q	P	P<0.05
12 Weeks vs. 6 Weeks	0.627	3	4.226	0.014	Yes
12 Weeks vs. 8 Weeks	0.222	3	1.494	0.547	No
8 Weeks vs. 6 Weeks	0.406	3	2.732	0.145	No

Comparisons for factor: **WEEK within VCD/STZ**

Comparison	Diff of Means	p	q	P	P<0.05
12 Weeks vs. 6 Weeks	0.493	3	3.323	0.062	No
12 Weeks vs. 8 Weeks	0.0528	3	0.355	0.966	Do Not Test
8 Weeks vs. 6 Weeks	0.441	3	2.968	0.104	Do Not Test

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

Statistics for Old Animals Treated with Rapamycin Study:

Student's Unpaired t-test Statistical Analysis for F4/80 Area:

Using the simple *t-test* found no significance in this experiment so a more appropriate Two-Way ANOVA was not performed. A difference with a $p<0.05$ was considered statistically significant. There was a low n of 3 in this study and since there are large variations in animal studies, more n is needed for this experiment to be conclusive.

ADULT						
Student's Unpaired t-test						
CONTROL	**LOW RAPA**		**CONTROL**	**HIGH RAPA**		**LOW RAPA**	**HIGH RAPA**
0.264	0.22		0.264	0.13		0.22	0.13
0.182	0.17		0.182	0.04		0.17	0.04
0.15	0.12		0.15	1.38		0.12	1.38
p =	0.5551		p =	0.5041		p =	0.4686

OLD							
Student's Unpaired t-test							
CONTROL	**LOW RAPA**		**CONTROL**	**HIGH RAPA**		**LOW RAPA**	**HIGH RAPA**
1.658	1.15		1.658	0.10		1.15	0.10
0.470	0.77		0.470	0.10		0.77	0.10
0.13	0.14		0.13	0.24		0.14	0.24
p =	0.9101		*p* =	0.2629		*p* =	0.1444

ADULT vs. OLD							
Student's Unpaired t-test							
CONTROL			**LOW RAPA**			**HIGH RAPA**	
ADULT	**OLD**		**ADULT**	**OLD**		**ADULT**	**OLD**
0.264	1.658		0.22	1.15		0.13	0.10
0.182	0.470		0.17	0.77		0.04	0.10
0.15	0.13		0.12	0.14		1.38	0.24
p =	0.2988		*p* =	0.1558		*p* =	0.4429

Student's Unpaired t-test Statistical Analysis for Interstitial Collage Area:

A *t-test* was the original analysis used for this data. A Two-Way ANOVA would not be appropriate because we looked only at the Old mice experimental group with one Adult Control group. Since there was significance found with the t-test between the Old and Adult Control a One-Way ANOVA may be appropriate.

* The *p*-values in **bold** denote statistical significance ($p<0.05$).

OLD							
Student's Unpaired t-test							
CONTROL	**LOW RAPA**		**CONTROL**	**HIGH RAPA**		**LOW RAPA**	**HIGH RAPA**
0.004523	0.006040		0.004523	0.0031790		0.006040	0.0031790
0.002432	0.001768		0.002432	0.0034990		0.001768	0.0034990
0.002809	0.001661		0.002809	0.0038380		0.001661	0.0038380
0.003959	0.005784		0.003959	0.0059315		0.005784	0.0059315
0.004189	0.003097		0.004189	0.0037714		0.003097	0.0037714
0.008187	0.002085		0.008187	0.0039975		0.002085	0.0039975
p =	0.4390		*p* =	0.7417		*p* =	0.5047

ADULT vs. OLD	
Student's Unpaired t-test	
CONTROL	
ADULT	**OLD**
0.001309	0.004523
0.001061	0.002432
0.001128	0.002809
	0.003959
	0.004189
	0.008187
***p* =**	**0.0355**

ANOVA on Ranks with Dunn's Post-Hoc Statistical Analysis for Interstitial Collagen Area:
The One-Way ANOVA failed because there were less data points in one of the groups compared to the others so the SigmaStat program continued with a One-Way ANOVA on Ranks. Although the ANOVA is more conservative than the *t-test*, both statistic analyses showed only one significant difference, $p<0.05$ between the Adult Control group and Old Control group.

One Way Analysis of Variance

Data source: Data 1 in Notebook

Normality Test: Failed (P = <0.001)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Notebook

Group	N	Missing	Median	25%	75%
ADULT CONTROL	3	0	0.00113	0.00108	0.00126
OLD CONTROL	6	0	0.00407	0.00281	0.00452
OLD LOW	6	0	0.00259	0.00177	0.00578
OLD HIGH	6	0	0.00380	0.00350	0.00400

H = 8.481 with 3 degrees of freedom. (P = 0.037)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.037)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (**Dunn's Method**):

Comparison	Diff of Ranks	Q	P<0.05
OLD CONTROL vs ADULT CONTROL	11.833	2.697	Yes
OLD CONTROL vs OLD LOW	3.500	0.977	No
OLD CONTROL vs OLD HIGH	0.500	0.140	Do Not Test
OLD HIGH vs ADULT CONTROL	11.333	2.583	No
OLD HIGH vs OLD LOW	3.000	0.837	Do Not Test
OLD LOW vs ADULT CONTROL	8.333	1.899	Do Not Test

Note: The multiple comparisons on ranks do not include an adjustment for ties.

Statistics for Old Animals Treated with Rapamycin Study:

Student's Unpaired t-test Statistical Analysis for Interstitial Collage Area:

Using the simple *t-test* found no significance in this experiment so a more appropriate One-Way ANOVA was not performed. A difference with a $p<0.05$ was considered statistically significant. There was a low n of 3 in this study and since there are large variations in animal studies, more n is needed for this experiment to be conclusive.

HCM/COXVII+/-	
Student's Unpaired t-test	
Female	**Male**
0.022045793	0.034511362
0.025346708	0.076757654
0.078433494	0.0272565
p =	0.8681

COXVII-/-	
Student's Unpaired t-test	
Female	**Male**
0.175226665	0.06721069
0.013083427	0.003279224
0.01876522	0.101258231
p =	0.8549

Female	
Student's Unpaired t-test	
HCM/COXVII+/-	**COXVII-/-**
0.022045793	0.175226665
0.025346708	0.013083427
0.078433494	0.01876522
p =	0.6549

Male	
Student's Unpaired t-test	
HCM/COXVII+/-	**COXVII-/-**
0.034511362	0.06721069
0.076757654	0.003279224
0.0272565	0.101258231
p =	0.7512

Both Sexes	
Student's Unpaired t-test	
HCM/COXVII+/-	**COXVII-/-**
0.022045793	0.175226665
0.025346708	0.013083427
0.078433494	0.01876522
0.034511362	0.06721069
0.076757654	0.003279224
0.0272565	0.101258231
p =	0.5280